

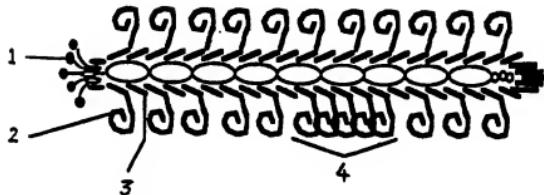
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(54) Title: ENGINEERED BACTERIOPHAGES AND VACCINES CONTAINING THEM



(57) Abstract

A filamentous bacteriophage includes, in at least a proportion of its major coat protein, a foreign peptide of at least 6 amino-acids that is capable of eliciting a biological response. For example, by incorporating an immunogenic peptide, an effective vaccine for malaria, foot-and-mouth disease and other infections can be obtained.

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ENGINEERED BACTERIOPHAGES AND VACCINES CONTAINING THEM
Field of the Invention

This invention relates to engineered bacteriophage and vaccines containing them.

5 Background of the Invention

The treatment of infectious diseases remains a considerable problem. Major efforts are being made to develop new and/or improved vaccines. For a variety of reasons, progress is slow, but the discovery that protective immunity can be obtained by administration of 10 only one of the constituent proteins of a pathogen, and in some cases only a fraction of protein, has offered new hope in the production of vaccines.

Foot-and-mouth disease virus (FMDV) belongs to the 15 picornaviridae, a family of positive-sense RNA viruses. FMDV remains a major scourge of live-stock in several continents. The control of this disease is either by slaughter, in those countries where FMDV does not normally occur, or by vaccination in endemic areas. The vaccines 20 that are currently in use are made from inactivated virus, which has been grown in tissue culture cells. This vaccine has proved to be very effective in controlling the disease, but there are many problems associated with handling large quantities of this highly infectious agent, and in ensuring 25 its complete inactivation. Moreover, these vaccines are difficult to administer in remote regions, and severe economic losses due to the disease still occur.

Another good example of a widespread disease, and 30 which has eluded immunologists for many years, is malaria. The major human malarial pathogen, *Plasmodium falciparum*, has a complex life-cycle and an ever-changing repertoire of surface antigens which allows highly effective evasion of the human immune-system. Numerous attempts have been made to control the disease but no satisfactory vaccine, 35 suitable for widespread administration, has been obtained.

Irradiated sporozoites have been used to immunize man, and protection against malaria has been achieved. However,

in this type of vaccine, both sexual and asexual forms of the parasite are required; sporozoites must, therefore, be prepared either from the salivary glands of infected mosquitoes or from cultured red blood cells. The former yields very small quantities, and the latter is a very expensive process.

The protective response to irradiated sporozoites has been localized to a protein (called the circumsporozoite protein) which is expressed on the surface of the organism and which does not mutate between generations. The most striking feature of this polypeptide chain is a large central repeat of the amino-acid sequence NANP (SEQ. ID No. 1), sometimes interspersed with the sequence NDVP (SEQ. ID No. 2). Further investigation has shown that twelve amino-acid units of this repeat sequence are sufficient for the production of antibodies reactive against the whole molecule. A wide variety of peptides, of twelve amino-acids or more, containing the repeat region of the circumsporozoite protein have been used to inoculate rabbits and mice; see, for example, Young *et al.*, Science 228 (1985) 985. Immunogenic responses have been obtained, but these are limited because oligopeptides do not generally provoke a humoral response.

Small peptides are poorly immunogenic, because they are missed by the cells of the immune-system or interact weakly with the cells they do meet. In addition, small peptides are excreted by the kidneys and, therefore, have only a short life-span in the blood-stream. In animal systems, some of these problems may be overcome by administering Freund's Complete Adjuvant (FCA) or aluminium hydroxide with the peptide, but both of these adjuvants are highly toxic and cannot be administered to humans. Peptides may be made more immunogenic if they are linked to a large carrier system after they have been synthesized, but this adds both to the complexity and expense of vaccine production.

To circumvent some of the problems associated with using small peptides as antigens, a different approach for vaccine production has been investigated. The genetic material of a virus is manipulated such that a foreign antigenic peptide is expressed on the surface of the virion. A DNA sequence which codes for the antigen is inserted into one of the coat protein genes; this may be a complex procedure, since many viruses contain RNA rather than DNA. For example, an antigenic epitope from polio virus type III has been inserted into the coat protein of tobacco mosaic virus; see Haynes *et al.*, Biotechnology 4 (1986) 637. A hybrid coat protein was produced and over-expressed in *E. coli* and, after purification, it could be assembled into virus-type rods. The hybrid TMV particles, injected into rats, were able to stimulate the production of antibodies which could neutralize polio virus. Unfortunately, this response was only obtained when FCA was employed.

Antibodies have also been expressed on the surface of attenuated animal viruses such as vaccinia, herpes and polio; see Dimmock *et al.* (ed.), *Foc. Gen. Microbiol. Symp.* 45, "Control of Virus Diseases", pub. Cambridge Univ. Press (1990). There are several problems associated with using animal viruses as vaccines: they are complex and their genetic material may be difficult to manipulate; they are difficult to prepare in large amounts and hence are expensive; they can sometimes revert to pathogenesis or exhibit some other form of toxicity.

Filamentous bacteriophages contain a single-stranded DNA genome which is encased within a protein sheath. The DNA becomes double-stranded after infection of the host strain, *Escherichia coli*, and both double- and single-stranded forms of DNA may be easily purified and manipulated (the virus M13, which is commonly used as a vector for site-directed mutagenesis and sequencing, is a filamentous bacteriophage). The genome encodes ten proteins, five of which are found in the mature phage

particle. Gene VIII encodes the major coat protein which forms the tubular structure of the phage capsid, genes VII and IX encode minor coat proteins which are located at one end of the phage particle, and genes III and VI encode 5 minor coat proteins positioned at the other end. A virus particle contains about five copies of each of the minor coat proteins.

The product of gene III is a large protein involved in 10 infection of the host cell. Chemically-synthesized DNA cassettes have been cloned into gene III and recombinant proteins have been produced; see de la Cruz *et al.*, J. Biol. Chem. 263 (1988) 4318. A number of recombinant proteins have been incorporated into viral particles and these were used to inoculate rabbits and mice, but in no 15 case did the immune-system respond with sufficient vigour to confer immunity. Although phages engineered in this way do not seem to be suitable for use as vaccines (since large and potentially dangerous doses would be required to elicit an effective response), this system is being developed for 20 production of "epitope-libraries" used, for example, to investigate the specificity of antibodies; see Scott & Smith, Science 249 (1990) 386.

Il'ichev *et al.*, Doklady Akademii Nauk SSR 307(2) (July 25 1989) 481-3, disclose phage M13 having a foreign peptide, 5 amino-acids long, incorporated into the major envelope protein. A "substantial effect on the infectiousness of the phage" is reported; this effect is actually a considerable decrease, to about 10%, of the reinfection potential. No other properties of the engineered virus 30 were reported.

Rowitsch *et al.*, J. Mol. Biol. 204 (1988) 663-674, disclose hybrid phage assembly.

Summary of the Invention

According to the present invention, a filamentous 35 bacteriophage includes, in at least a proportion of its major coat protein, a foreign peptide of 6 or more amino-acids that is capable of eliciting a biological response.

The present invention is based on the realisation that the failure of the gene III fusion proteins in vaccine production is almost certainly due to the low number of proteins found in each phage and/or to the masking of the epitope by the bulk virus structure. The major coat protein (the product of gene VIII), however, is found as about 2500 copies per virus, and peptides having an immunogenic effect have now been inserted into this protein. Moreover, they are expressed in a region of the coat protein known to be exposed on the surface of the virus. As indicated below, the coat protein containing the foreign peptide may desirably be present in balance with unmodified (wild-type) virus coat protein.

Description of the Drawings

Figure 1 is a schematic view of a hybrid filamentous bacteriophage. It shows a gene III protein 1, a coat protein with epitope 2, a wild-type coat protein 3, and an epitope 4 that is too large for normal packing of subunits.

Figure 2 shows the construction of plasmid pKfdH. The process involves isolating the NlaIV fragment and then the SnaB1/NlaIV fragment, followed by ligation into SmaI/phosphatase-treated pKK223-3.

Description of the Invention

A bacteriophage of the invention includes a foreign peptide: the peptide may be antigenic, e.g. any that raises a desired immunological response, e.g. against a form of the malaria parasite or against another virus such as foot-and-mouth disease virus. It may already be known for this purpose. Its length should be sufficient to raise the response but insufficient to modify the bacteriophage's properties undesirably or to prevent its incorporation, e.g. at least 9 amino-acids.

A bacteriophage of the invention is preferably immunogenic, and is suitable for use in vaccines, and generally as a therapeutic/diagnostic product. Therefore, products of the invention may be formulated with any

suitable physiologically-acceptable diluent or carrier, to prepare a vaccine composition.

In addition, by using two or more compatible vectors to carry suitably engineered coat protein genes, two or 5 more differently modified coat proteins can be assembled into the same virion, to enable more than one peptide to be displayed simultaneously. This can lead to the generation of multi-shot vaccines and to the generation of more complex structures on the surface of the virion with 10 correspondingly complex biological properties.

A potential disadvantage of this or other viruses as vaccines is that they contain DNA, which may be regarded as undesirable for injection into the animal being protected. With the filamentous bacteriophage carrier described here, 15 the DNA within the virion may be selectively degraded by dialysis at acid pH before injection.

In an alternative embodiment of the invention, the foreign peptide is an agonist/antagonist of a cell receptor (e.g. a biologically-active peptide such as a hormone, 20 releasing factor or growth factor) or an inhibitor of an enzyme (e.g. an inhibitor of renin or the AIDS virus proteinase) or an enzyme (phage enzyme) or an antibody (phage antibody) or any other protein or peptide with a desired biological activity. For some purposes, the 25 peptide may include a non-metabolisable component. Bacteriophages including a variety of such peptides can be used to screen for preferred structure and activity. In addition, the hybrid phage system has other broad applications. By inserting varied or random DNA sequences 30 to encode the peptides to be displayed, an infinite number of peptides can be expressed on the surface of the virion, thus creating "epitope libraries".

Epitope libraries allow large numbers (sometimes tens of millions) of small peptides to be surveyed for any 35 desirable property, e.g. tight binding to an antibody, receptor or other binding protein. For example, while antibodies can be purified from the serum of a patient with

an auto-immune disease, the target of the antibodies is often unknown. An epitope library can be used to find the antigenic target of the antibodies, hence identifying the physiological site of the disease and suggesting a route
5 for therapeutic procedures. It may also be possible to discover peptides which mimic the biologically-active regions of hormones, cytokines, enzyme substrates, neuropeptides and other biomolecules. Peptides which could either replace or alter the action of the natural ligands
10 would be powerful candidates for drug development. The hybrid phage allows the number of copies of a peptide displayed on each viral particle to be controlled within wide limits, and can confer great sensitivity.

The major coat protein of the filamentous
15 bacteriophage fd is encoded by gene VIII. The protein is synthesized as a procoat which contains a 23 amino-acid leader-peptide attached to the N-terminus of the mature protein. After synthesis, the procoat protein is rapidly inserted into the inner-membrane of *E. coli* where it is processed to leave the 50 amino-acid mature coat protein spanning the membrane. This protein has three domains, a hydrophobic membrane-spanning domain, a positively-charged C-terminal domain which faces into the cytoplasm of the cell, and a negatively-charged N-terminal domain which extends into the periplasm.
20 During assembly of virus articles, the coat protein subunits are pulled out of the cell membrane and become arranged in a helical array around the viral DNA. In the viral particle, the C-terminal region of the protein subunits faces inwards towards the
25 DNA such that the positively-charged residues may neutralize the negative charges of the sugar-phosphate backbone. The N-terminal domain, by contrast, is on the outer surface of the particle where it is exposed to the environment. Nuclear magnetic resonance studies have
30 indicated that this region is flexible both in the membrane-bound form of the protein and when presented on the outside of the phage particle. This region is also
35

known to be highly antigenic, antibodies prepared against whole phage particles being directed almost solely towards this domain of the coat protein. In this invention, peptides are attached to the outside of the phage coat 5 protein, to give extended coat proteins that successfully assemble into viral particles and elicit a strong immune-response.

Therefore, in order to prepare an embodiment of the invention, epitopes are inserted into the N-terminal domain 10 of the major coat protein of bacteriophage fd, such that they decorate the outside of an assembled phage particle. This problem may be approached entirely at the DNA level, by engineering a restriction enzyme recognition site into gene VIII, and allowing insertion of oligonucleotide 15 cassettes, encoding the desired epitope, directly into the phage genome. In order to study this problem in a controlled manner, however, it was preferred to separate the synthesis of any hybrid coat proteins from the assembly 20 of phage particles. This allows the cloning and expression of novel coat proteins, and the membrane-insertion and packaging stages of phage assembly, to be studied as independent entities.

In order to allow independent expression of the fd 25 coat protein gene, gene VIII may be subcloned into a controllable expression vector such as pKK223-3 which uses the lac promoter. However, the wild-type gene VIII does not contain a suitable restriction enzyme site for the 30 insertion of oligonucleotide cassettes (which would be used to encode the epitopes). Therefore, before the gene is subcloned, a suitable site may be introduced by means of site-directed mutagenesis. By way of example, this site 35 was blunt-ended (to allow "in-frame" insertion of DNA), was positioned between the third and fourth codons of the mature coat protein (in order to minimize disruption of the leader-peptidase recognition sequence in the procoat) and was recognised by HpaI which does not have any recognition sites in pKK223-3, thus providing a unique cloning site.

In Preparing a hybrid bacteriophage of the invention, an intermediate product is a plasmid encoding the modification. In order to prevent the loss of such a plasmid, it is preferred that the engineered coat protein 5 is transplanted into a phage, phagemid or other replicon, with a packaging signal. The recombinant gene can then be packaged and selected for further replication.

The following Examples illustrate the preparation and utility of products of this invention. With the exception 10 of the methods A, B and C described below, all experimental procedures are as described by Greenwood *et al.*, J. Mol. Biol. 220 (1991) 821-827; see also Rowitsch *et al* (1988), *supra*.

A Polyacrylamide gel electrophoresis using the tricine 15 buffer system

Proteins were separated on denaturing polyacrylamide gels composed of: 49.5% (w/v) acrylamide/ 0.5% (w/v) bisacrylamide (7 ml); 3 M Tris HCl, pH 8.4 (7 ml), glycerol (4.2 ml) and H₂O (2.8 ml), set with 25% (w/v) ammonium 20 persulphate (200 µl) and TEMED (20 µl). The stacking gel contained: 49.5% (w/v) acrylamide/ 0.5% (w/v) bisacrylamide (1 ml), 3 M Tris HCl, pH 8.4 (2.5 ml) and H₂O (6.8 ml), set with 25% (w/v) ammonium persulphate (100 µl) and TEMED (10 µl). The gel cassette measured 17 cm x 17 cm and was 1 cm 25 thick.

Samples were loaded in 4 times their volume of denaturing buffer, i.e. DTT (154 mg), 10% (w/v) SDS (2 ml), glycerol (1 ml), 1 M Tris HCl, pH 6.8 (170 µl), H₂O (1.63 ml, 0.2% (w/v) Bromophenol blue in ethanol (200 µl). 30 Samples were heated at 100°C for 2 min before loading onto gels. The running buffer contained: 1.8% (w/v) Tricine, 1.2% (w/v) Tris base, and 1% (w/v) SDS.

Gels were run overnight at 100 V or during the day at 35 250 V until the bromophenol blue dye front reached the bottom.

B Electroblotting of proteins onto PVDF membrane

Proteins were separated using tricine-PAGE as described in Method A with the addition of 2 mM mercaptoacetic acid in the upper electrode buffer (used to scavenge N-blocking free radicals). When the gel had run to completion it was soaked in transfer buffer (25 mM Tris base, 190 mM glycine, 10% (v/v) MeOH) for 10 min. The PVDF membrane was prepared by immersion in MeOH for 10 s followed by equilibration in transfer buffer for 5 min. The 5 proteins were electroblotted onto the PVDF membrane using a Biorad transblot apparatus. Transfer was carried out at 150 mA for 3-16 h with cooling to 16°C. When transfer was 10 complete the PVDF was immediately soaked in water for 10 min with shaking. The membrane was rinsed in water and was 15 allowed to air-dry thoroughly.

C Detection of proteins on Western Blots using alkaline phosphatase

Proteins were electrophoretically transferred onto nitrocellulose. The membrane was soaked in TBST buffer (10 mM Tris HCl, pH 8.0, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20) 20 for 10 min, followed by the same buffer containing 1% (w/v) BSA for 20 min. The membrane was washed with TBST (without BSA) for 10 min. The membrane was soaked in TBST containing antibody directed against bacteriophage fd or 25 the hybrid bacteriophage (1 µl antibodies/1 ml TBST) for 30 min and was then washed in TBST 3 times (10 min per wash). After washing, goat anti-rabbit IgG-alkaline phosphatase-conjugate was added (1 µl antibodies/1 ml TBST) and incubation was continued for 30 min. The membrane was 30 washed 3 times as before and the blot was developed in AP buffer (100 mM Tris HCl, pH 9.0, 100 mM NaCl, 5 mM MgCl₂) containing about 1.6 mg nitro blue tetrazolium and about 0.8 mg 5-bromo-4-chloro-3-indolyl phosphate. Both of the substrates could be prepared as stocks in water shortly 35 before use.

Example 1

1.1 Site-directed mutagenesis and subcloning of fd gene VIII

An 18mer oligonucleotide (SEQ ID No. 3) was
5 synthesized on a Biosearch 8600 machine using
phosphoamidite chemistry, and was purified by
polyacrylamide gel electrophoresis. Single-stranded DNA
template was prepared from wild-type fd and mutagenesis was
performed using the phosphorothioate method and available
10 in kit form from Amersham International plc. A number of
bacteriophages were selected and sequenced using a second
oligonucleotide (SEQ ID No. 4, synthesized and prepared as
above) which binds towards the 3'-end of gene VIII. A
phage was isolated which bore the correct mutation and was
15 designated fdH.

Insertion of the HpaI site led to a change in the
amino-acid sequence of the mature coat protein (SEQ ID No.
5) from Gly-Asp at positions 3 and 4 to Val-Asn (SEQ ID No.
6). Such a change clearly did not significantly affect the
20 membrane-insertion and processing of the procoat protein,
nor the assembly of the coat protein into bacteriophage
particles, since mutant phage were viable and could be
propagated in yields comparable with the wild-type.

The scheme for subcloning the fdH gene VIII is shown
25 in Figure 2. Double-stranded RF DNA of fdH was prepared
and digested with the blunt-end cutter NlaIV to generate a
number of fragments including a 292 bp fragment which
contained gene VIII. This band was isolated by
polyacrylamide gel electrophoresis and was further digested
30 with a second blunt-end cutter, SnaBI, which removed 20
base pairs upstream of the gene (already known to interfere
with efficient expression of the wild-type gene). The
shortened fragment was again purified by polyacrylamide gel
electrophoresis and was cloned into pKK223-3 which had been
35 cut with SmaI and treated with calf intestine alkaline
phosphatase. Recombinants which contained the fdH gene in

the correct orientation for expression were identified by restriction digests and were designated pKfdH.

1.2 Expression of the fdH gene

Expression of the fdH gene was tested *in vivo* using *E. coli* strain TGL (*lacIq*) transformed with plasmid pKfdH. Transformed cells were grown in YT medium supplemented with 50 µg ampicillin/ml at 37°C to an A_{600} value of approximately 0.3, and were then induced with IPTG (final concentration 250 µM). At various times after induction samples of cells were removed, collected by centrifugation, washed and analysed by means of polyacrylamide gel electrophoresis using the tricine buffer system (tricine-PAGE). Staining the gels with silver revealed that before induction there was no detectable coat protein produced; after induction, transformed cells contained increasing amounts of a protein which had an electrophoretic mobility similar to that of wild-type coat protein. In these *in vivo* experiments, the procoat protein is being inserted into the cell innermembrane and is being processed by leader-peptidase. In the case of the fdH protein, the mutations clearly had not interfered with either of these processes and there were large amounts of processed coat protein available in the cell membrane.

Therefore, the insertion of the *Hpa*I site and the concomitant change in the primary structure of the procoat protein did not interfere with bacteriophage assembly. The mutated coat protein was successfully cloned into the controllable expression vector pKK223-3 where its synthesis could be induced at will. The procoat protein synthesized from the plasmid was also competent for membrane-insertion and was correctly processed.

Although it was perhaps unsurprising that the insertion of the *Hpa*I site into fd gene VIII had little effect on the membrane-insertion and processing of the slightly altered procoat protein, the addition of a number of extra amino-acids into this small protein was a novel and more dramatic procedure, and the effects on membrane-

insertion could not be predicted. Failure at this stage would obviously prohibit assembly of the fusion coat proteins into viral particles.

The second stage of the work, therefore, was to insert 5 an oligonucleotide cassette into the HpaI site and to investigate the effects on the membrane-insertion and processing of the resulting procoat protein. The chosen epitope (SEQ ID No. 7) was the repeat sequence of the Plasmodium falciparum coat protein (SEQ ID No. 1). This 10 was known to elicit an immune response when administered as a synthetic peptide and, at only 12 amino-acids, was one of the smallest well-defined epitopes.

1.3 Insertion of DNA cassettes into the fdH gene

Two oligonucleotides were synthesized and purified as 15 described in Section 1.1. One was SEQ ID No. 7, encoding (NANP)₃, and the other was the complementary sequence (SEQ ID No. 8). To produce the double-stranded DNA cassette, equal amounts of these two oligonucleotides were heated to 67°C and allowed to cool slowly to room temperature. The 20 36 bp cassette was ligated into pKfdH DNA which had been treated with HpaI and calf intestine alkaline phosphatase. Ligated plasmids were transformed into E. coli strain JM109 (lacIq, rec 0). Recombinants could first be identified by restriction analysis since successful insertion of DNA 25 would destroy the HpaI site. Plasmids which were found to be resistant to digestion with HpaI were sequenced in order to determine the number and orientation of inserted cassettes. A clone was isolated that contained one copy of the 36 bp cassette in the correct orientation for expression and was designated pKfdMal.

1.4 Expression of the fd fusion protein fdMal *in vivo*

E. coli cells (strain JM109) harbouring plasmid pKfdMal are induced with IPTG exactly as described for cells harbouring pKfdH (Section 1.2). Before induction 35 only E. coli proteins were present; after induction, increasing amounts of a protein appeared which was considerably larger than the wild-type fd coat protein.

Estimation of the molecular weight of the protein from polyacrylamide gel electrophoresis (approx. 7 kDa) was consistent with it being the fdMal fusion protein. Moreover, this result strongly suggested that the procoat 5 fusion protein had been inserted into the cell membrane and correctly processed. Western-blotting of proteins from the induced cells revealed that the fusion protein was not recognised by antibodies raised against wild-type fd phage.

As a result, a general scheme for the production of 10 fusion proteins in E. coli has been created; a gene containing an additional 36 base-pairs has been successfully cloned and expressed. The fusion protein contains twelve amino-acids inserted into the centre of the procoat protein (a 16% increase in size of the mature 15 region), but these extra residues did not interfere with membrane-insertion of the protein. The leader-peptide was efficiently removed from membrane-bound procoat, indicating that the inserted epitope did not disrupt the leader-peptidase recognition sequence. Antibodies raised against 20 wild-type fd phage did not recognise the fusion protein, suggesting that this system allows immunogenic specificity.

It is known from previous work that fd coat protein 25 expressed from a plasmid in a non-suppressor strain of E. coli can support the growth of a bacteriophage, R252, that has an amber mutation in its own coat protein gene. In addition, it has been possible to produce 'hybrid' bacteriophage containing both wild-type coat proteins expressed from the phage genome and mutant coat proteins expressed from a plasmid within the host cell. This was 30 possible even when the mutant coat protein could not support phage growth in the absence of wild-type coat protein. It has, therefore, been possible to study the ability of the fdMal coat protein to assemble into bacteriophage particles both in isolation and when 35 'diluted' with wild-type coat proteins.

1.5 Production of hybrid bacteriophages

E. coli strain JM109 (lacIq, su) transformed with plasmid pKfdMal was grown at 37°C with shaking in YT medium containing ampicillin (50 µg/ml) until a A_{600} value of -0.3 was reached. The cells were then infected with either 5 phage fd or phage R252, growth was continued for 20 min and cells were induced with IPTG (10 µM final concentration). Growth was allowed to continue overnight. Phage were harvested and purified as described by Greenwood *et al* (1991) supra, and samples were analysed by tricine-PAGE 10 followed by staining with silver.

The yield of R252 phage was very poor and electrophoretic analysis revealed that the only coat protein present was of the same mobility as wild-type coat protein. The small amount of phage produced was therefore 15 assumed to be due to the low level of reversion and pseudoreversion that occurs within the population of amber mutant phages.

The yield of fd phage, on the other hand, was good (approx. 10 mg purified phage from 1 l of culture). 20 Electrophoretic analysis revealed the presence of two coat proteins, one with the same mobility as wild-type and one with the same mobility as the fdMal protein expressed from pKfdMal. These two proteins were present in almost equal amounts (there is only slightly more of the wild-type coat 25 protein).

1.6 Characterisation of the fdMal protein

For absolute confirmation of the identity of the larger protein, a sample was separated from the wild-type protein by electrophoresis and was transferred onto PVDF 30 membrane (method B). The N-terminal sequence was determined as SEQ ID No. 9, i.e. AEV(NANP)₃N. This indicated that the complete peptide had been inserted into the expected position in the coat protein and that normal processing of the procoat had occurred.

It was therefore apparent that the addition of twelve 35 amino-acids into the N-terminal domain of the mature coat protein of the phage was incompatible with phage assembly

unless the wild-type coat protein was also present. The most likely explanation for this is that the extra peptide is too bulky to allow acceptable packing of the coat proteins where the fusion proteins alone constitute the coat; however, when wild-type coat proteins are also present, these may act as 'spacers' allowing the fusion proteins to be incorporated. This has been represented diagrammatically in Figure 1. Although this adds slightly to the complexity of making phage containing fusion proteins (since it would clearly be futile to try to insert the oligonucleotide cassette directly into the phage genome), it may, in fact, be an advantage for the production of antibodies or other biologically-active peptide inserts, since the peptide is allowed more space to fold into the correct 3-dimensional structure, facilitating the production of an immune or other specific biological response.

There a number of important criteria for the successful use of the fdMal hybrid phages in vaccines. For example, it is necessary for a good immune response to be achieved without the addition of stimulants, such as Freund's Adjuvant or aluminium hydroxide, if the phage are to be injected into humans. It is important that the malaria epitope within the coat protein be available to the immune-system and that any antibodies produced against the fusion protein can also recognise the epitope when presented in a different form, such as the free peptide in solution (and eventually as part of the malaria parasite). Finally, it is important that the antibodies react specifically with the malaria epitope.

1.7 Production of antibodies

New Zealand White rabbits were injected subcutaneously with 0.5 mg samples, at 1 mg/ml, of either wild-type bacteriophage fd or wild-type/fdMal hybrid bacteriophages. Rabbits were injected on days 1, 14, 28, 38, 42, 56 and 66. No other compounds were administered. After 6 weeks the rabbits were bled out and the serum was isolated.

1.8 Western blot analysis

Samples of wild-type phage and wild-type/fdMal hybrid phage were subjected to tricine-PAGE and were electrophoretically transferred to nitrocellulose. Western blots were prepared using either anti-fd or anti-fd/fdMal antibodies. The anti-fd antibodies strongly recognised the wild-type fd coat protein but did not recognise the fdMal protein. Anti-fd/fdMal antibodies recognised both the fd protein and the fdMal protein. This was expected since both proteins are present in the hybrid phage which were injected into rabbits. However, the reaction against the fdMal protein was noticeably stronger than the reaction against the wild-type protein. This suggested two things: (1) that the larger fusion proteins were either more antigenic than the fd coat proteins or that they were shielding the wild-type proteins from the immune-system, so that there were more antibodies produced against fdMal than against fd; (2) that the anti-fdMal antibodies were not recognising the wild-type coat proteins. Both of these factors suggested that the hybrid phage had generated a response predominantly directed against the malaria epitope attached to the phage rather than the phage themselves.

1.9 Recognition of synthetic peptides

The antibodies generated against the fd/fdMal hybrid phage clearly reacted against the fdMal fusion protein; however, it was important that it could also recognise the malaria epitope when it was presented in a different form. The easiest way of testing for this was to check reactivity against an (NANP)₃ peptide that had been chemically synthesized.

In fact, synthesis of (NANP)₃ itself is technically difficult because the C-terminal proline residue causes premature termination. To circumvent these difficulties and extra amino-acid (asn = N) was added to the C-terminal end. Samples of purified (NANP)₃N (see SEQ ID No. 10) were spotted onto nitrocellulose and were allowed to dry. For comparison, a second unrelated peptide (SEQ ID No. 11), a

sample of phage fd and a sample of phage fd/fdMal were treated in a similar fashion. These samples were incubated with either anti-fd antibody (1:1000 dilution) or anti-fd/fdMal antibody (at dilutions of 1:500, 1:1000, 1:2000, 5 1:5000). After washing the filters, goat anti-rabbit IgG-alkaline phosphatase-conjugate was added. Following further washing, the filters were developed by addition of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

10 The antibodies raised against bacteriophage fd reacted with both the fd and fd/fdMal hybrid phage samples. These antibodies did not cross-react at all with either the synthetic (NANP)₃N peptide or with the control peptide. Antibodies raised against fd/fdMal hybrids, however, 15 reacted not only with both pure and hybrid phage samples, but also very strongly with the synthetic (NANP)₃N peptide. No reaction was seen with the control peptide.

Injection of hybrid bacteriophage into rabbits, without the addition of an immunostimulant, such as 20 Freund's Complete Adjuvant, was sufficient to generate a strong immune-response. A high titre of antibodies was produced and these reacted strongly with a sample of hybrid bacteriophage. The mixed population of antibodies had a great affinity for the malaria fusion protein, could 25 recognise a synthetic version of the malaria peptide and did not cross-react with an unrelated peptide. Hence, the immune-response was both vigorous and very specific.

In summary of Example 1, a malaria parasite peptide has been inserted into the protein capsid of bacteriophage 30 fd; the vigorous production of antibodies in rabbits and the ability of the antibodies to cross-react strongly with a synthetic malaria peptide have been demonstrated.

Example 2

35 In this Example, oligonucleotides were designed and subcloned into gene VIII DNA to enable a region of the 141-160 epitope from FMDV Vp1 to be displayed on the outside of the phage particles.

2.1 Making fd/fd FMDV hybrid bacteriophage

Oligonucleotide cassettes (SEQ ID Nos. 12 and 13) were synthesised. These encode the region 141-154 in FMDV Vp1. The oligonucleotides were treated with the enzyme 5 polynucleotide kinase, annealed and ligated into pKfdH (as previously described). Plasmids containing the cassettes in the correct orientation were screened for by DNA sequencing. Hybrid bacteriophage were made (as described above) which contained a ratio of wild-type to extended 10 coat protein of 3:1. Rabbits were inoculated with these bacteriophage; serum was isolated and assayed using an ELISA system. Either bacteriophage at 0.1 µg/ml or chemically-synthesised peptide SEQ ID No. 14 cross-linked to keyhole limpet haemacyanin was adsorbed onto 96-well 15 plates in 0.1M sodium carbonate buffer, pH 8.3. After 16 hours at room temperature the unoccupied sites were blocked by the addition of 5% bovine serum albumin in Tris-buffered saline and 0.1% Tween 20 (TBST) for 2 hours at room 20 temperature. The rabbit serum was then added to the plates at a range of dilutions.

After 30 min at room temperature, the serum was removed and the plates washed with TBST. The plates were incubated for 30 min with goat anti-rabbit IgG cross-linked to alkaline phosphatase at 1:1000 dilution. The plates 25 were washed to remove excess secondary antibody and the presence of the alkaline phosphatase located using an AMPAK system (Novo Bio).

The rabbit serum was found to react strongly with both the hybrid bacteriophage and the chemically-synthesised 30 peptide, showing that the rabbit was responding to the epitopes displayed on the outside of the bacteriophage.

2.2 Testing the fd/FMDV bacteriophage as a vaccine

Guinea pigs (4 in each of 5 groups plus control animals) were inoculated with the fd/FMDV hybrid 35 bacteriophage and various adjuvants. The animals were then challenged with live virus.

The amounts of phage, the adjuvants and the results are given in the following Table (FICA = Freund's Incomplete Adjuvant). The results clearly illustrate that the fd/fd FMDV hybrid bacteriophage provide protection
 5 against FMDV.

	Dose	Adjuvant	Protection	Lesion
10	0	-	0/4	12
	500 µg	PBS	2/4	2
15	500 µg	FICA	4/4	0
	500 µg	Al(OH) ₃	4/4	0
20	50 µg	FICA	2/4	5
	50 µg	Al(OH) ₃	1/4	8

In summary, Example 2 demonstrates that, by displaying epitopes on the surface of bacteriophage, it is possible to create an highly effective vaccine to FMDV. This vaccine
 30 has many powerful advantages over those that are already commercially available. It is very stable and does not require refrigeration. It is extremely cheap and easy to produce. Moreover, a wide range of different epitopes can be displayed on the surface of the bacteriophage to create
 35 a vaccine that can protect against number of serotypes. This technology can be used to develop vaccines against numerous infectious diseases including AIDS.

Example 3

A unique *Hpa*I-restriction site was engineered between
 40 the codons specifying residues 3 and 4 of the mature coat protein (to generate vector fdH), and an oligonucleotide

encoding the hexapeptide YGFWGM (SEQ. ID No. 15) was cloned at this site into the bacteriophage fdH RF DNA. Viable progeny were isolated in all cases. On SDS-polyacrylamide gel electrophoresis, the lowered mobility of the enlarged 5 coat protein was consistent with the insertion of an additional 6-amino-acids, and its amino-acid composition was found to be similarly consistent. However, it did not prove possible to insert more than 6 amino-acids directly into the major coat protein of bacteriophage fd by this 10 technique. This might be due to one or more of several reasons: instability of the modified protein, a failure of membrane-insertion and processing of the novel procoat molecule, or an inability subsequently to package the DNA.

22

SEQUENCE LISTING

SEQ ID No. 1

Sequence Type: Peptide

Sequence Length: 4 amino-acids

Original Source Organism: Plasmodium falciparum

Asn Ala Asn Pro

SEQ ID No. 2

Sequence Type: Peptide

Sequence Length: 4 amino-acids

Original Source Organism: Plasmodium falciparum

Asn Asp Val Pro

SEQ ID No. 3

Sequence Type: Nucleotide

Sequence Length: 18 bases

Strandedness: Single

Topology: Linear

GGGATCGTTA ACCTCAGC

23

SEQ ID No. 4

Sequence Type: Nucleotide

Sequence Length: 17 bases

Strandedness: Single

Topology: Linear

CCATCGCCCCA CGCATAA

SEQ ID No. 5

Sequence Type: Peptide

Sequence Length: 9 amino-acids

Original Source Organism: Bacteriophage fdh

Feature: Ala Ala Leader-Peptidase recognition site

Ser	Phe	Ala	Ala	Glu	Gly	Asp	Asp	Pro
-1	1					5		

SEQ ID No. 6

Sequence Type: Peptide

Sequence Length: 9 amino-acids

Original Source Organism: Bacteriophage fdh

Feature: Val Asn Insertion site

Ser	Phe	Ala	Ala	Glu	Val	Asn	Asp	Pro
-1	1					5		

24

SEQ ID No. 7

Sequence Type: Nucleotide with corresponding peptide

Sequence Length: 36 bases

Strandedness: Single

Topology: Linear

AAT GCA AAC CCG AAC GCA AAC CCG AAT GCA AAC CCG
Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro
5 10

SEQ ID No. 8

Sequence Type: Nucleotide

Sequence Length: 36 bases

Strandedness: Single

Topology: Linear

CGG GTT TGC ATT CGG GTT TGC GTT CGG GTT TGC ATT

SEQ ID No. 9

Sequence Type: Peptide N-terminal

Sequence Length: 16 amino-acids

Ala Glu Val Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn
5 10 15

25

SEQ ID No. 10

Sequence Type: Peptide

Sequence Length: 13 amino-acids

Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn
5 10

SEQ ID No. 11

Sequence Type: Peptide

Sequence Length: 11 amino-acids

Ala Ala Pro Ala Ala Ala Pro Ala Pro Ala Ala
5 10

SEQ ID No. 12

Sequence Type: Nucleotide

Sequence Length: 36 bases

Strandedness: Single

Topology: Linear

CGG CAG GGT GCG CGC CAC TTT CTG CGC CAG CAC CTG

SEQ ID No. 13

Sequence Type: Nucleotide

Sequence Length: 36 bases

Strandedness: Single

Topology: Linear

CTG CGC CAG CAC CTG CAG ATC GCC GCG CAG GTT CGG

SEQ ID No. 14

Sequence Type: Peptide

Sequence Length: 19 amino-acids

Val Pro Asn Leu Arg Gly Asp Gln Val Leu Ala Gln Lys Val Ala
5 10 15
Arg Thr Leu Pro

SEQ ID No. 15

Sequence Type: Nucleotide with corresponding peptide

Sequence Length: 18 bases

Original Source Organism: N-terminal peptide of β -endorphin

TAT GGT TTC TGG GGT ATG
Tyr Gly Phe Trp Gly Met
5

CLAIMS

1. A filamentous bacteriophage including, in at least a proportion of its major coat protein, a foreign peptide of at least 6 amino-acids that is capable of eliciting a biological response.
2. A bacteriophage according to claim 1, wherein the peptide has at least 9 amino-acids.
3. A bacteriophage according to claim or claim 2, wherein the peptide is an agonist or antagonist of a cell receptor or an enzyme inhibitor.
4. A bacteriophage according to claim 1 or claim 2, wherein the peptide is antigenic or immunogenic.
5. A bacteriophage according to claim 4, wherein the peptide raises a response against malaria.
- 15 6. A bacteriophage according to claim 4, wherein the peptide raises a response against foot-and-mouth disease.
7. A bacteriophage according to any preceding claim, whose DNA is degraded.
- 20 8. A vaccine composition comprising a bacteriophage according to any of claims 4 to 7, in association with a physiologically-acceptable carrier or diluent.
9. A method for preparing a bacteriophage according to any of claims 1 to 6, which comprises introducing a unique restriction enzyme site into gene VIII, subcloning the thus modified gene VIII into a controllable expression vector, inserting a cassette encoding the peptide into the vector, and assembling the protein product of the resultant vector into the wild-type bacteriophage.
- 25 10. A method according to claim 9, wherein the gene encoding the engineered coat protein is transplanted into a replicon including a packaging signal.

1/1

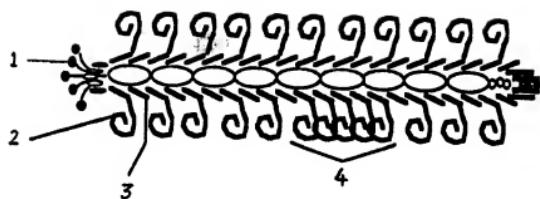


Fig. 1

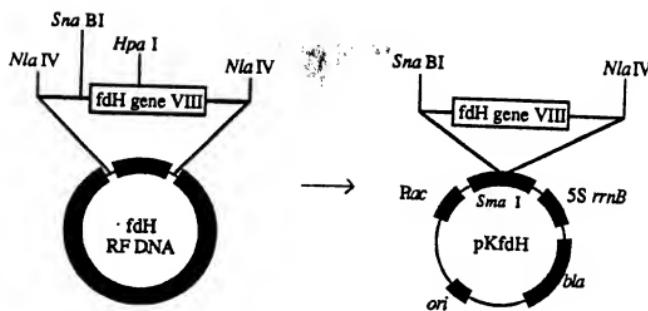


Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 91/01785

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)^a

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/34;	C12N7/01;	C12N15/42;	C12N15/30
C12N15/70;	A61K39/015;	A61K39/12	

II. FIELDS SEARCHED

Minimum Documentation Searched^b

Classification System	Classification Symbols	
Int.Cl. 5	C12N ;	C07K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched^cIII. DOCUMENTS CONSIDERED TO BE RELEVANT^d

Category ^e	Citation of Document, ^f with indication, where appropriate, of the relevant passages ^g	Relevant to Claim No. ^h
P, X	J. MOL. BIOL. vol. 220, August 1991, LONDON, UK pages 821 - 827; J. GREENWOOD ET AL.: 'Multiple Display of Foreign Peptides on a Filamentous Bacteriophage' cited in the application see the whole document ----	1-5, 7-10
Y	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 263, March 1988, BALTIMORE US pages 4318 - 4322; V. F. DE LA CRUZ ET AL.: 'Immunogenicity and Epitope Mapping of Foreign Sequences via Genetically Engineered Filamentous Phage' cited in the application see abstract ----	2-5, 8 ----

^a Special categories of cited documents:ⁱ^b "A" document defining the general state of the art which is not considered to be of particular relevance^c "E" earlier document but published on or after the international filing date^d "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)^e "O" document referring to an oral disclosure, use, exhibition or other means^f "P" document published prior to the international filing date but later than the priority date claimed^g "T" later document published after the international filing date or priority date and not to conflict with the application but cited to understand the principle or theory underlying the invention^h "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive stepⁱ "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art^j "A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

2

15 JANUARY 1992

03.02.92

International Searching Authority

Signature of Authorized Officer

EUROPEAN PATENT OFFICE

THIELE U.H.-C.H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	NATURE. vol. 330, 26 November 1987, LONDON GB pages 381 - 384; B. E. CLARKE ET AL: 'Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein' see abstract	6
X	CHEMICAL ABSTRACTS, vol. 111, no. 19, 6 November 1989, Columbus, Ohio, US; abstract no. 171037, A. A. ILL'ICHEV ET AL.: 'Production of the M13 phage viable variant with a foreign peptide inserted into the coat basic protein' page 427 ;column L ; cited in the application	1
Y	see abstract & DOKL. AKAD. NAUK. SSSR vol. 307, no. 2, 1989, MOSKOW, RUSSIA pages 481 - 483;	2-6,8